acidified with HCl to give 1.8 g (98%) of pale-yellow crystals, mp 211-212 °C, after recrystallization from CH_3OH-EtOH. Anal. ($C_{19}H_{17}Cl_2NO_3$·HCl) C, H, Cl, N.

6,7-Dichloro-1-(3,4,5-trimethoxybenzyl)-1,2,3,4-tetrahydroisoquinoline (6b) Fumarate. A suspension of 11·HCl (1.6 g, 3.9 mmol) in H_2O was converted to the base (10 N NaOH, CH_2Cl_2, H_2O wash, $MgSO_4$), which was dissolved in 5 mL of THF and treated with 18 mL of 0.97 M BH_3 in THF. After the solution was stirred at reflux under N_2 for 6 h, an additional 5 mL of the BH_3 solution was added, and refluxing and stirring were continued for an additional 1.5 h under N_2. The solution was cooled to 10 °C; CH_3OH was added dropwise with *caution*, and then the solution was concentrated. To the residue was added 20 mL of 2 N HCl, and the solution was refluxed for 30 min. After the solution was cooled to 20 °C it was made alkaline with 10 N NaOH, and the mixture was extracted with EtOAc. The EtOAc layer was washed with H_2O, dried ($MgSO_4$), and concentrated. The residue was dissolved in CH_3OH, and excess fumaric acid in CH_3OH was added. Et_2O was added to precipitate a solid (0.6 g, 50%), mp 163 °C, after recrystallization from EtOH. Anal. ($C_{19}H_{21}Cl_2NO_3$·$C_4H_4O_4$·0.25H_2O) C, H, N.

Determination of Distribution Coefficients. The compounds were partitioned by shaking between *n*-octanol and pH 7.4 phosphate buffer (prepared from 3.7 g of Na_2HPO_4 and 1.6 g of KH_2PO_4 in sufficient H_2O to make 9 L, adjusting the pH to 7.4 by addition of 0.02 M KH_2PO_4 or 0.01 M Na_2HPO_4 as required) until equilibrium was attained. The phases were allowed to separate and were clarified by centrifugation. Aliquots from each phase were then assayed by UV detection. The compounds were partitioned at four different concentrations, and the ratios (D = (milligrams of compound/milliliters of *n*-octanol)/(milligrams of compound/milliliters of pH 7.4 phosphate buffer)) were plotted vs. concentration. The value of D (or log D) was determined at infinite dilution by linear regression.

pK_a determinations were obtained by titration of the compounds in 1:2 CH_3OH-H_2O (to overcome solubility constraints).

True partition coefficient, P, was calculated from the distribution coefficient D as follows: $P = D/(1 - \alpha)$, where $\alpha = 1/[1 + \text{antilog}(\text{pH} - \text{p}K_a)]$.

Pharmacology. Inhibition of Isoproterenol-Induced Enhancement of Rate of Contraction of Spontaneously Beating Guinea Pig Atrial Pairs. Adult, male Hartley strain guinea pigs weighing 500-800 g were anesthetized with sodium pentobarbital (100 mg/kg, ip). The whole heart was quickly removed and immersed in 10 °C Krebs solution of the following concentration: NaCl (119 mM), $CaCl_2$ (2.5 mM), KH_2PO_4 (1.2 mM), KCl, 4.7 mM), $NaHCO_3^-$ (25 mM), glucose (11.1 mM), ascorbic acid (5 μM), and disodium EDTA (30 μM). The atrial pair was dissected free of the rest of the heart tissue and fat and was suspended via surgical thread secured to the apex of each atria in 50-mL tissue baths containing Krebs solution maintained at 37 °C and gassed continuously with a mixture of 95:5 O_2-CO_2. Cocaine (6 μM) was present throughout all experiments. Atrial contractions were recorded with a Grass FT 03D isometric force displacement transducer on a Beckman R-511 dynograph; atrial rate was recorded on the same instrument from cardiotachometers triggered by the atrial contractions.

The atrial pair was allowed to equilibrate for approximately 30 min prior to administration of test compounds. During this equilibration period the bathing solution was exchanged three or four times. A concentration-response curve to isoproterenol was then determined, increasing isoproterenol concentrations when the rate response to the previous dose had stabilized. After this determination, any β-agonist activity of a test compound was measured via a concentration-response curve. To determine β-antagonist activity, a concentration of test compound that produced no β response (3 μM for **6b** and propanolol, which lacked agonist activity at concentrations as high as 100 μM) was allowed to remain in the tissue bath while the curve for isoproterenol was repeated. The receptor dissociation constant (K_B) as a β-adrenergic antagonist was determined by using the following formula: K_B = antagonist concentration/(dose ratio - 1), where the dose ratio is the EC_{50} (the concentration increasing the rate of contraction to 50% of maximal for compound being tested) for isoproterenol in the presence of antagonist divided by the EC_{50} for isoproterenol. The reported K_B values are the average of 4-8 experiments.

Acknowledgment. We are grateful to Edith A. Reich for determination of elemental analyses and to Dr. Walter Holl and Dr. R. Lee Webb for physicochemical determinations.

Registry No. **6b**·$C_4H_4O_4$, 103959-65-5; **7**, 75416-52-3; **8**·HCl, 73075-60-2; **9**, 103959-61-1; **10**, 103959-62-2; **11**·HCl, 103959-63-3; 3,4,5-trimethoxybenzyl chloride, 3840-30-0.

Synthesis and Biological Evaluation of Irreversible Inhibitors of Aldose Reductase

Jeffrey J. Ares,*[†] Peter F. Kador,[‡] and Duane D. Miller*[†]

Division of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, The Ohio State University, Columbus, Ohio 43210, and Laboratory of Mechanisms of Ocular Disease, National Eye Institute, National Institutes of Health, Bethesda, Maryland 20892. *Received May 28, 1985*

5-Isothiocyanatoalrestatin (**1b**) and 5-azidoalrestatin (**1c**) were prepared synthetically and examined as potential affinity and photoaffinity inhibitors of rat lens aldose reductase. Both compound **1b** and **1c** under appropriate conditions at 10^{-4} M produced a 70% irreversible inactivation of aldose reductase within 1 min. The enzyme could, in part, be protected by preincubation with sorbinil **2**, a known potent inhibitor of aldose reductase.

The enzyme aldose reductase uses NADPH as a cofactor to reduce glucose to sorbitol. This reaction accounts for the first step of the polyol pathway, which subsequently leads to the formation of fructose. Although the polyol pathway has no known function in man, the enzyme aldose reductase is important because intracellular sorbitol accumulation occurs in the presence of excess glucose in various tissues and is linked to many of the disabling complications of diabetes including cataracts, retinopathy, corneal wound healing, and neuropathy.[1-3] These complications are believed to result in part from sorbitol accumulation, which leads to osmotic swelling and disturbances in cell function.[1] For this reason, inhibitors of aldose reductase are clinically desirable for reduction of sorbitol accumulation and possible alleviation of these diabetic complications.

Many structurally diverse compounds have been shown to inhibit aldose reductase. Among the more potent are

[†] The Ohio State University.
[‡] National Institutes of Health.

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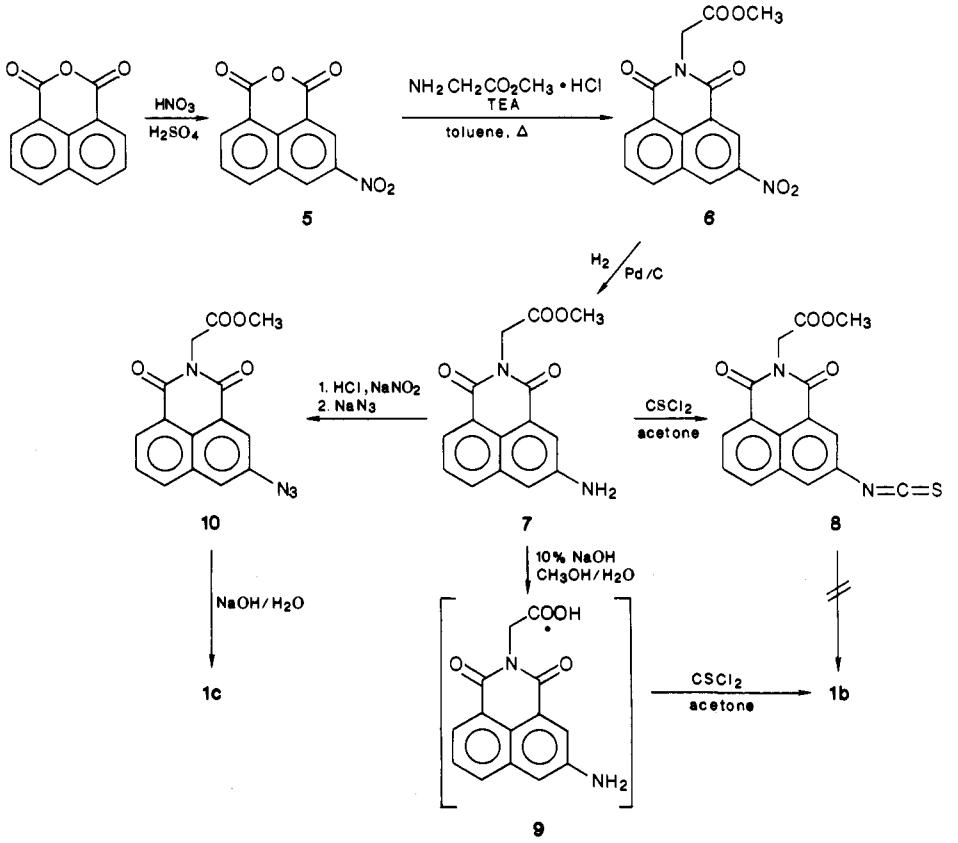
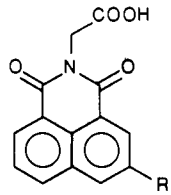
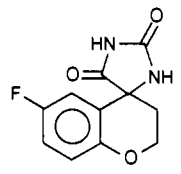
Scheme I



alrestatin (**1**), sorbinil (**2**), tolrestat (**3**), and ONO-2235 (**4**).[4-7] Kinetic studies have indicated that these inhibitors



1a, R = H
b, R = N=C=S
c, R = N_3



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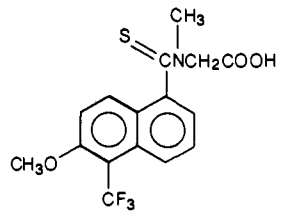

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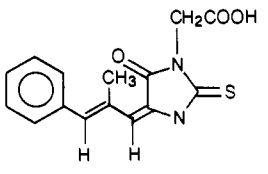

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interact with the enzyme at a site independent of either the substrate or nucleotide cofactor site, with inhibition obeying noncompetitive or uncompetitive kinetics.[2,3,8] It has been proposed that three distinct regions are present on the enzyme: a substrate site, a nucleotide fold, and an inhibitor site.[3,8] A thorough understanding of this inhibitor binding site would aid in the rational design of new and more potent aldose reductase inhibitors.

Preliminary efforts to characterize the aldose reductase inhibitory binding site has led to the suggestion that a nucleophilic group, capable of reversible interaction with the inhibitor, is present at this site.[8] Such a nucleophilic group may be expected to interact irreversibly with an appropriately electrophilic functional group attached to a molecule with known affinity for the inhibitor binding site. This concept, known as affinity labeling, has been extensively employed in the characterization of various enzyme binding sites and receptors.[9-11] A similar concept, that of photoaffinity labeling, can be utilized if an appropriately reactive nucleophile is either absent or, for steric or electronic reasons, unable to interact with an affinity label.[12] Therefore, it appears reasonable that the inhibitory binding site of aldose reductase may be an excellent candidate for affinity and photoaffinity labeling. Experiments of this type would determine if aldose reductase could be irreversibly inactivated via the inhibitory binding site and would provide a foundation for future attempts to "map" this site. Here, we report the synthesis and biological evaluation of the 5-isothiocyanato (**1b**) and 5-azido (**1c**) derivatives of alrestatin as potential affinity and photoaffinity labels, respectively, of aldose reductase.

Chemistry. The preparation of labels **1b** and **1c** was carried out as indicated in Scheme I. Nitration of 1,8-

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naphthalic anhydride was carried out by modification of a procedure of Anselm and Zuckmayer.[13] The nitro anhydride 5 was then condensed with glycine methyl ester hydrochloride in refluxing toluene, which generated, after catalytic hydrogenation, the common intermediate amino ester 7. When 7 was treated with thiophosgene in acetone, isothiocyanate 8 was obtained, but attempts to remove the methyl protecting group by various hydrolytic and demethylating procedures such as BBr_3 and $(CH_3)_3SiI$ failed to generate the desired acid **1b**. Isothiocyanate **1b** could be prepared by base hydrolysis of 7 to an intermediate amino acid **9**, which was not isolated but was treated directly with thiophosgene in acetone to provide the desired compound **1b**. Preparation of azide **1c** proceeded in a straightforward manner by first generating the azido ester **10** via diazonium salt formation. Base-catalyzed hydrolysis of **10** provided the light-sensitived azido acid 1c.

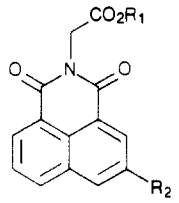
Biological Results and Discussion

The wide structural diversity of the many aldose reductase inhibitors reported in the literature has been noted by Kador and Sharpless.[8] These inhibitors compete with neither substrate nor cofactor and thus are felt to interact with the enzyme at a common inhibitor binding site. This binding site has been suggested to contain a nucleophilic amino acid, a hydrogen bonding residue, and a hydrophobic pocket.[8] The possible presence of either a nucleophilic amino acid or a hydrogen bonding residue, which may itself be nucleophilic, makes the aldose reductase inhibitor binding site an attractive candidate for affinity labeling. A proposed hydrophobic pocket, however, might lack a nucleophilic handle. Therefore, an in situ generated reactive species, i.e., a photoaffinity label, would be an appropriate compound to analyze as well.

Alrestatin (**1a**) is a noncompetitive reversible inhibitor of aldose reductase.[14] Since our affinity and photoaffinity labels are structural modifications of this reversible inhibitor, it was first necessary to clearly demonstrate that enzyme inactivation by our analogues was irreversible and due to the presence of the additional functional group. This differentiation was accomplished via gel filtration chromatography with a BioRad P-6DG desalting gel packed in disposable polypropylene BioRad columns, which serves to rapidly separate enzyme from any reversibly bound or unreacted drug. This column is highly effective in separating potent reversible inhibitors such as sorbinil from the enzyme and, as such, provided a useful means of carrying out time-dependent irreversible inactivation studies, as well as protection studies by means of label concomitantly incubated with reversible aldose reductase inhibitors.

Labels **1b** and **1c**, as well as their methyl ester analogues 8 and **10**, displayed concentration-dependent irreversible inactivation of aldose reductase as determined by overnight incubation with enzyme (and photolysis in the case of the azides). The mixtures were subjected to P-6DG chromatography to remove any unreacted affinity label, and remaining enzyme activity was photometrically assayed with glyceraldehyde as substrate. The concentrations of drug necessary for 50% inhibition (IC_{50}'s) were estimated from regression analysis as previously described.[15,16] All four agents demonstrate the ability to irreversibly inhibit aldose reductase in a concentration-dependent manner, and in the case of both the affinity and photoaffinity label, esterification of the carboxylic acid reduces inhibitor activity by approximately 1 order of magnitude (Table I). This, coupled to the observation that most reversible aldose reductase inhibitors contain an acidic functional group (carboxylic acid, phenol, or imide), suggests that an ionizable portion of the molecule is important in binding to the inhibitor site. Kinetic analyses by Lineweaver–Burke and Hanes–Wolf plots indicated that the inhibition of **1b**, **1c**, 8, and **10** was noncompetitive with respect to glyceraldehyde and NADPH cofactor.

Table I. Inhibition (IC_{50}'s) of Purified RLAR by Alrestatin Analogues[a]



compd	R_1	R_2	IC_{50}, μM
1b	H	N=C=S	0.3
1c	H	N_3	3.6
8	CH_3	N=C=S	14.0
10	CH_3	N_3	38.0

[a] Determinations were performed in duplicate with each determination representing 4–8 cuvette measurements. IC_{50} values were obtained as described in the Experimental Section using either a Guilford Response or Guilford 2400-2 spectrophotometer.

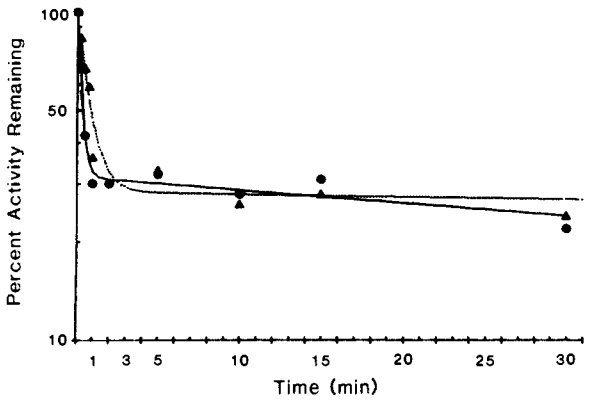


Figure 1. Time-dependent irreversible inactivation of RLAR by isothiocyanate **1b** (●) and azide 1c (▲). The azide is photolyzed prior to determination as described in the Experimental Section.

Time-dependent inactivation of aldose reductase by 1b and **1c** could also be demonstrated as indicated in Figure 1. Incubation of isothiocyanate **1b** followed by aliquot removal over intervals up to 30 min and BioRad P-6DG gel filtration chromatography resulted in a dramatic rapid loss of enzyme activity over the first minute followed by a gradual waning of enzyme activity over the next half hour. Incubation and photolysis of azide **1c** followed by similar aliquot removal and chromatography also resulted in rapid enzyme inactivation over 1 min followed by an activity plateau over the remaining 20 min (Figure 1). The inability of these agents to completely inactivate the enzyme is suggestive of a maximum degree of conformational regulation that the inhibitor site exerts on catalytic activity.

True affinity and photoaffinity labeling of the aldose reductase inhibitor site may be suggested if known inhibitors, possessing affinity for the binding site, can suitably protect the enzyme from irreversible alkylation. Therefore, protection studies were conducted and they

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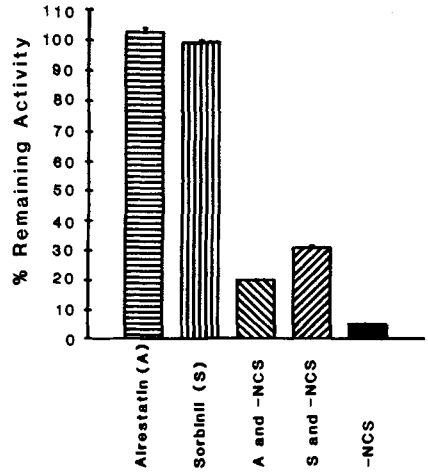

Figure 2. Protection of isothiocyanate-induced inactivation of RLAR by reversible AR inhibitors, alrestatin and sorbinil (mean ± SEM, n = 4–8).

Table II. Irreversible Inhibition of Purified RLAR

	% inhibn	
compd (10 μM)	alone	preincubated with 100 μM sorbinil
1b	57	5
phenyl isothiocyanate	34	5

suggest that labeling does occur at a common binding site (Figures 2 and 3). Preincubation of enzyme with either alrestatin (**1a**) or sorbinil (**2**), followed by treatment with isothiocyanate (**1b**) and then BioRad P-6DG chromatography, demonstrated enzyme protection relative to the degree of inactivation observed with the isothiocyanate alone (Figure 2). It is noteworthy in this experiment that BioRad chromatographic techniques can completely remove the reversible inhibitors alrestatin and sorbinil to regenerate enzyme activity, as illustrated with alrestatin (A) and sorbinil (S) in Figures 2 and 3. To further demonstrate the specific nature of this irreversible inhibition, the 10-min incubation of 10^{-5} M isothiocyanate **1b** dissolved in 2.5% Me_2SO was compared to phenylisothiocyanate (Table II). Preincubation with 10^{-4} M sorbinil reduced the irreversible inhibition with both compounds to 5%.

Similar protection experiments for the azide **1c** are shown in Figure 3. Here sorbinil (**2**) is much more effective than alrestatin (**1a**) in accomplishing enzyme protection. The significance of this lies in the fact that the concentrations of protecting agents were very small, i.e., the same as the concentration of the label used in the experiment. Sorbinil's ability to protect in a superior manner to alrestatin is a reflection of its greater potency in inhibiting aldose reductase as compared to alrestatin.[3] This figure also demonstrates the inability of the azide to irreversibly block AR in the absence of photolysis.

Thus, it is possible to irreversibly inactivate aldose reductase through the use of an affinity or photoaffinity label, based upon the reversible inhibitor alrestatin. This report serves to confirm the earlier report of Kador and Sharpless,[8] which first suggested the presence of a nucleophilic amino acid. Tyrosine was initially suggested, based on the use of protein modification reagents.[8] It is unknown, however, whether the photoaffinity label is binding the same nucleophilic amino acid as the affinity label or possibly a less reactive hydrophobic amino acid that may be part of the hydrophobic pocket. It is anticipated that these studies may aid in the rational design

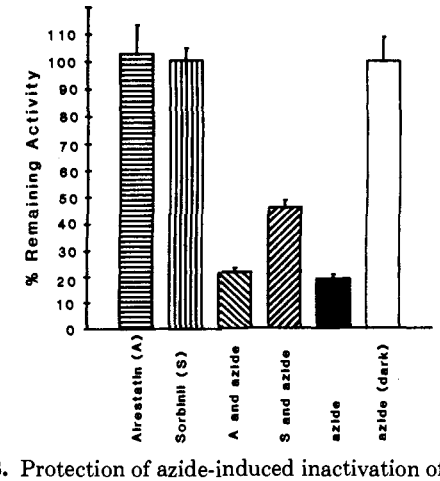

Figure 3. Protection of azide-induced inactivation of RLAR by reversible inhibitors, alrestatin and sorbinil (mean ± SEM, n = 4–8).

of more potent and selective inhibitors of aldose reductase. These are the first studies reported on the inactivation of aldose reductase using affinity labels on the inhibitor binding site.

Experimental Section

Melting points (uncorrected) were determined on a Thomas-Hoover melting point apparatus. Spectral data were obtained with a Beckman 4230 infrared spectrophotometer and a Bruker HX-90E NMR spectrometer (90 MHz) in pulse mode. Mass spectra were obtained at The Ohio State University Chemical Instrument Center with a Kratos-MS 30 mass spectrometer. Analyses were performed by Galbraith Laboratories, Inc., Knoxville, TN. Analytical results for elements indicated were within ±0.4% of the theoretical values.

3-Nitro-1,8-naphthalic Acid Anhydride (5). Coupound **5** was prepared by modification of a procedure of Anselm and Zuckmayer[13] as follows: 5 g (0.025 mol) of commercially available 1,8-naphthalic acid anhydride was dissolved in 20 mL of concentrated H_2SO_4 and cooled in an ice bath to 5 °C. Meanwhile, a solution of 1.57 g (0.025 mol) of concentrated HNO_3 in 5 mL of concentrated H_2SO_4 was prepared and added slowly via dropping funnel to the cooled naphthalic anhydride solution with stirring, such that the reaction mixture does not exceed 20 °C. After addition was complete, the ice bath was removed, and the mixture was allowed to warm to room temperature and stir for 90 min. The reaction mixture was then poured into ice water, and the solid so obtained was filtered by suction, washed well with water, and recrystallized from glacial acetic acid. Anhydride **7** crystallized as yellow needles giving 4.4 g (73%) of solid: mp 243 °C [lit. 249 °C]; NMR ($CDCl_3$, 90 MHz) δ 9.48 (d, J = 2.2 Hz, 1 H), 9.13 (d, 1 H), 8.92–8.75 (m, 2 H), 8.16 (m, 1 H).

5-Nitro-1,3-dioxo-1*H*-benz[*de*]isoquinoline-2(3*H*)-acetic Acid Methyl Ester (6). Nitro anhydride **5** (9.02 g, 0.037 mol), glycine methyl ester hydrochloride (4.64g, 0.037 mol), triethylamine (8 mL), and toluene (450 mL) were placed in a 1-L round-bottom flask equipped with a condenser and a Dean-Starke trap and were heated to reflux for a period of 6 h. The brown reaction mixture was then decolorized immediately with activated charcoal and filtered while hot. Solvent removal in vacuo left a residue, which was recrystallized from acetone to yield **6** as a colorless solid obtained in two crops: 9.2 g (79%); mp 203–205 °C; NMR ($CDCl_3$, 90 MHz) δ 9.33 (d, J = 2.2 Hz, 1 H), 9.16 (d, 1 H), 8.81 (d of d, J = 1.3 Hz and 7 Hz, 1 H), 8.46 (d of d, J = 1.3 Hz and 8.5 Hz, 1 H), 7.98 (m, 1 H), 4.97 (s, 2 H), 3.80 (s, 3 H); mass spectrum, m/e 314.05 (M^+). Anal. ($C_{15}H_{10}N_2O_4$) C, H, N.

5-Amino-1,3-dioxo-1*H*-benz[*de*]isoquinoline-2(3*H*)-acetic Acid Methyl Ester (7). Ester **6** (1.25 g, 3.98 mmol) was dissolved in 175 mL of ethyl acetate with gentle heating. After cooling, the solution was added to a Parr bottle containing 350 mg of 5% Pd/C. The mixture was then hydrogenated at 40 psi for 3 h. Filtration of catalyst by gravity through double filter paper gave a greenish solution, which, upon solvent removal in vacuo, yielded

a greenish yellow solid. Recrystallization with ethyl acetate provided **7** as a bright-yellow solid: 833 mg (74%); mp 230–231 °C; IR (KBr) 3470, 3380 (NH_2) cm^{-1}; NMR ($CDCl_3$,90 MHz) δ 8.37–7.28 (m, 5 H), 4.93 (s, 2 H), 4.23 (br s, exchangeable with D_2O, 2 H), 3.78 (s, 3 H); mass spectrum, *m/e* 284.08 (M^+). Anal. ($C_{15}H_{12}N_2O_4$) C, H, N.

5-Isothiocyanato-1,3-dioxo-1*H*-benz[*de*]isoquinoline-2-(3*H*)-acetic Acid Methyl Ester (8). Thiophosgene (172 mg, 1.5 mmol) was dissolved in 28 mL of acetone and stirred in an ice bath in a hood. Meanwhile, **7** (420 mg, 1.4 mmol) was dissolved in 70 mL of acetone and added slowly via dropping funnel to the cooled thiophosgene solution, with the reaction mixture stirring vigorously. After addition, the mixture was stirred in ice for 1 h and then at room temperature for 30 min. Water was then added dropwise to the reaction mixture to force the product out as a solid, which was recrystallized from acetone/water to provide **8**: 256 mg (56%); mp 180 °C; IR (KBr) 2020 (br, N=C=S) cm^{-1}; NMR ($CDCl_3$, 90 MHz) δ 8.65–7.72 (m, 5 H), 4.95 (s, 2 H), 3.79 (s, 3 H); mass spectrum, *m/e* 326.04 (M^+). Anal. ($C_{16}H_{10}N_2O_4S$) C, H, N, S.

5-Isothiocyanato-1,3-dioxo-1*H*-benz[*de*]isoquinoline-2-(3*H*)-acetic Acid (1b). Ester **7** (100 mg, 0.352 mmol) was suspended in 10 mL of methanol to which 6 mL of 10% aqueous NaOH solution was added. The resulting solution was stirred at room temperature for 18 h, after which time a suspension had formed. Methanol was removed in vacuo, and the resulting mixture was carefully neutralized to pH 7 with 10% HCl, forming a solution. Meanwhile, 121 mg (1.05 mmol) of thiophosgene was dissolved in 25 mL of acetone and cooled in ice. The amino acid solution was then added dropwise to the thiophosgene. After addition, the mixture was stirred in ice for 1 h and at room temperature for an additional 1 h. Removal of acetone in vacuo resulted in crystallization of a product, which was recrystallized from acetone to afford 72 mg (65%) of the desired acid: mp 228–230 °C dec; IR (KBr) 3550–2700 (br, COOH), 2060 (br, N=C=S) cm^{-1}; NMR (acetone-d_6, 90 MHz) δ 8.63–7.86 (m, 5 H), 4.88 (s, 2 H), 2.88 (br s, 1 H); mass spectrum, *m/e* 312.016 (M^+). Anal. ($C_{15}H_8N_2O_4S$) C, H, N, S.

5-Azido-1,3-dioxo-1*H*-benz[*de*]isoquinoline-2(3*H*)-acetic Acid Methyl Ester (10). Ester **7** (1 g, 3.5 mmol) was mixed with 4 mL of concentrated HCl and 4 mL of water in a flask equipped with a dropping funnel and a thermometer. The solution was cooled to 0 °C in a salt–ice bath, and then a solution of 720 mg (0.104 mol) of sodium nitrite in 5 mL of water was added dropwise with stirring. The mixture was then stirred in the salt–ice bath for 1 h with the temperature maintained at 0 to −5 °C. Meanwhile, a solution of 340 mg (5.23 mmol) of sodium azide in 3 mL of water was placed in a beaker and cooled in ice. The diazonium salt solution was then added to the cooled NaN_3 solution with stirring. As the nitrogen evolved away, the product formed as a crystalline material, which was filtered and washed well with water. decolorization with activated charcoal and recrystallization with EtOAc provided **10** as a white, fluffy, light-sensitive solid, 609 mg (56%): mp 181 °C dec; IR (KBr) 2110 (sharp, N_3) cm^{-1}; NMR ($CDCl_3$, 90 MHz) δ 8.57–7.67 (m, 5 H), 4.95 (s, 2 H), 3.79 (s, 3 H); mass spectrum, *m/e* 310.072 (M^+). Anal. ($C_{15}H_{10}N_4O_4$) C, H, N.

5-Azido-1,3-dioxo-1*H*-benz[*de*]isoquinoline-2(3*H*)-acetic Acid (1c). The azido ester **10** (1.6 g, 5.16 mmol) was suspended in 500 mL of CH_3OH and heated gently on a steam bath to near dissolution. Then 100 mL of 10% aqueous NaOH solution was added to the warm mixture, which resulted in complete dissolution. The solution was then stirred at room temperature for 15 min, followed by slow acidification to pH 3 with 10% HCl. Removal of methanol in vacuo resulted in crystallization of the product, which was filtered. The product was then dissolved in warm acetone, followed by treatment with activated charcoal, filtration, and removal of acetone in vacuo. The purified product was a light-sensitive yellow solid, 720 mg (47%): mp 194–6 °C dec; IR (KBr) 3500–2700 (br, COOH), 2115 (sharp, N_3) cm^{-1}; NMR (acetone-d_6, 90 MHz) δ 8.51–7.77 (m, 5 H), 4.88 (s, 2 H), 2.70 (br s, 1 H); mass spectrum, *m/e* 296.057 (M^+). Anal. ($C_{14}H_8N_4O_4$) C, H, N.

Preparation of RLAR. Purified aldose reductase from rat lens was prepared by affinity chromatography on Amicon Matrex Gel Orange A by a modification of the procedure previously described[17] in which 20 mM potassium phosphate buffer, pH 7.5, containing 7 mM mercaptoethanol, 0.5 mM EDTA, and 10% glycerol (v/v) were used as buffer.[18] Frozen eyes from adult Sprague-Dawley rats of mixed sex were obtained from Biotrol, Indianapolis, IN. After thawing, the lenses were carefully removed by posterior approach, and all subsequent steps were conducted at 22 °C. Eight hundred lenses were homogenized with 600 mL of 20 mM potassium phosphate buffer, pH 7.5, containing 7 mM mercaptoethanol, 0.5 mM EDTA, and 10% glycerol (v/v), in a 40-mL Wheaton Dunce tissue grinder, and the homogenate was centrifuged at 12000*g* for 10 min. To the resulting stirred supernatant was added solid ammonium sulfate to give a 40% saturation. After centrifugation at 12000*g* for 10 min, the pellet was discarded and the supernatant increased to 70% saturation by the addition of solid ammonium sulfate. Following centrifugation at 12000*g* for 10 min, the precipitate was dissolved in a small amount of 20 mM potassium phosphate buffer, pH 7.5, containing 7 mM mercaptoethanol, 0.5 mM EDTA, and 10% glycerol (v/v) and dialyzed against 2 × 2 L of the same buffer solution. The dialysate was placed on a 2.5 × 45 cm BioRad column containing 150 mL of Amicon Matrex Gel Orange A equilibrated with the same buffer. The column was eluted with the same buffer and collected in 300-drop aliquots (about 18.0 mL). After 37 fractions had been collected, the column was eluted with the same buffer containing 0.1 mM NADPH. Fractions containing enzyme activity were concentrated on a Amicon column concentrator equipped with a YM-10 membrane to an approximate volume of 3 mL and stored at 4 °C. The enzyme appeared as a single band, MW 38 K on SDS-PAGE.

Enzyme Assay. Aldose reductase activity was spectrophotometrically determined by following the decrease in NADPH concentration at 340 nm on either a Guilford Response or 2400-2 spectrophotometer as previously decribed.[15] Kinetic analyses with glyceraldehyde and NADPH were conducted as previously described, using the Binkin2 public procedure of the NIH PROPHET computer system.[16]

Preparation of Enzyme Prior to Alkylation. The purified enzyme was dialyzed against 0.1 M Na,K-phosphate buffer (pH 7.2) by gel filtration through a PD-10 column containing Sephadex G-25M (Pharmacia Fine Chemicals).

Determination of Apparent IC_{50}'s of Alkylated Enzyme. To purified enzyme in a glass scintillation vial containing 0.1 M phosphate buffer, pH 7.2, at 4 °C was added either the acid or ester isothiocyanate or azide dissolved in Me_2SO to give an appropriate 10^{-4}, 10^{-5}, or 10^{-6} M solution in 3 mL total volume containing 5% Me_2SO. Controls contained only 5% Me_2SO solutions. The isothiocyanates were then incubated overnight (approximately 12 h) at 4 °C with magnetic stirring, while the stirred azides were subjected to overnight photolysis from a Sylvania DWC 150 W flood light approximately 24 in. from the glass. Unreacted drug was then removed from a 2.5-mL aliquot of the enzyme solution by gel filtration through a PD-10 column with 3.5 mL of 0.1 M phosphate buffer, pH 7.2. Enzyme activity of the filtrate was photometrically assayed with glyceraldehyde as substrate.[15]

Competition Studies. To equal aliquots of purified enzyme in 0.1 M phosphate buffer was added either acid azide, acid isothiocyanate, alrestatin, or sorbinil (all carefully dissolved in dilute base) to give a 10^{-4} M aqueous solution of all drugs in a volume of 1.0 mL. Controls contained water instead of drug. Solutions of alrestatin and sorbinil were preincubated for 30 min pritor to the addition of either azide or isothiocyanate. The isothiocyanate solutions were then incubated overnight at 4 °C with stirring, while the azide solutions were subjected to 45-min photolysis from the flood lamp 12 in. away. After the appropriate time periods 0.75-mL enzyme aliquots were placed on 3.1-mL BioRad P-6DG desalting gel packed in disposable 0.8 × 4 cm polypropylene BioRad columns. The solutions were then eluted with 1.0 mL of 0.1 M phosphate buffer, pH 6.2, and enzyme activity was spectrophotometrically monitored with 10 mM glyceraldehyde as described above except that the enzyme solution

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also contained 0.2 M lithium sulfate.

Incubation with Phenyl Isothiocyanate (PITC). To equal aliquots of purifed enzyme in 0.1 M phosphate buffer was added either phenyl isothiocyanate (enzyme modification grade, Pierce Chemical Company) or **1b** dissolved in Me_2SO to give a 10^{-5} M solution containing 2.5% Me_2SO. Controls contained Me_2SO instead of drug. Aqueous solutions of 10^{-4} M sorbinil were incubated 30 min prior to the addition of isothiocyanate. The isothiocyanate solutions were incubated for 10 min at 4 °C with stirring prior to gel filtration and assay as described above.

Timed Studies. To equal aliquots of purified enzyme in 0.1 M phosphate buffer was added either acid azide or acid isothiocyanate to give a 10^{-4} M aqueous solution of all drugs in a volume of 4.0 mL. Controls contained water instead of drug. Upon stirred incubation or photolysis from a flood lamp at a distance of 12 in., 0.75 mL-aliquots of enzyme solution were removed at intervals of 15 s to 30 min. These aliquots were immediately placed onto a 3.1-mL BioRad P-6DG desalting gel packed in disposable 0.8 × 4 cm polypropylene BioRad columns and then eluted with 1.0 mL of 0.1 M phosphate buffer, pH 6.2. Enzyme activity was spectrophotometrically monitored with 10 mM glyceraldehyde as described above in the presence of 0.2 M lithium sulfate.

All results were the average of 4–8 cuvette measurements on either a Guilford Response or Guilford 2400-2 spectrophotometer.

Registry No. **1b**, 103904-10-5; **1c**, 103884-86-2; **5**, 3027-38-1; **6**, 103884-82-8; **7**, 103884-83-9; **8**, 103884-84-0; **10**, 103884-85-1; Gly-OMe·HCl, 5680-79-5; 1,8-naphthalic acid anhydride, 81-84-5; aldose reductase, 9028-31-3.

9-(2-Deoxy-2-fluoro-β-D-arabinofuranosyl)guanine: A Metabolically Stable Cytotoxic Analogue of 2′-Deoxyguanosine

John A. Montgomery,*† Anita T. Shortnacy,† Dennis A. Carson,‡ and John A. Secrist III*†

Kettering-Meyer Laboratory, Southern Research Institute, Birmingham, Alabama 35255-5305, and Department of Clinical Research, Scripps Clinic and Research Foundation, La Jolla, California 92037. *Received December 23, 1985*

The synthesis of 9-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)guanine (**1b**) from 1,3-di-*O*-acetyl-5-*O*-benzoyl-2-deoxy-2-fluoro-D-arabinofuranose (**2a**) and 2,6-dichloropurine in six steps using an enzymatic deamination as the last step is reported. The target compound was found to be stable to purine nucleoside phosphorylase cleavage and was cytotoxic in two cell lines, one a T-cell line. Incubation of L1210 cells with **1b** results in an inhibition of DNA synthesis as judged by the reduced incorporation of labeled thymidine into DNA, while RNA and protein syntheses were unaffected.

The enhanced toxicity of certain deoxynucleosides toward T-cell lines relative to B-cell lines may result from the greater ability of these cells to accumulate toxic concentrations of the corresponding deoxynucleoside triphosphates.[1-4] Certain immunodeficiency diseases are associated with deficiencies of adenosine deaminase or purine nucleoside phosphorylase,[5-9] which result in increased pools of deoxynucleoside triphosphates. Recent reports indicate that 9-β-D-arabinofuranosylguanine (*ara*-G, **1a**), an analogue of 2′-deoxyguanosine, preferentially inhibits DNA synthesis in T- relative to B-lymphoblasts and is a selective inhibitor of T-lymphoblast growth.[10-14] *ara*-G is readily metabolized to the triphosphate level in various cell lines[10-15] and has been found to inhibit DNA polymerase α from L5178Y mouse lymphoma cells. In the presence of *ara*-G these cells die because of unbalanced growth.[15] *ara*-G has been reported to be resistant to purine nucleoside phosphorylase when tested against the isolated enzyme and in vitro,[10,14] but two reports indicate that it is degraded in vivo.[10,16]

Analogues with the basic properties of *ara*-G are attractive as potential chemotherapeutic agents for T-cell malignancies. We have now prepared another such analogue of 2′-deoxyguanosine, 9-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)guanine (**1b**) and carried out an initial examination of its cytotoxicity and inhibitory effects. The known resistance of 9-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)hypoxanthine to cleavage by purine nucleoside phosphorylase[17] made **1b** a particularly attractive target.

Chemistry. The synthesis of **1b** appeared to be best accomplished by the condensation of a 2-deoxy-2-fluoro-arabinose derivative with a purine. Starting with 1,3-di-*O*-acetyl-5-*O*-benzoyl-2-deoxy-2-fluoro-β-D-arabinose (**2a**),[18,19] we examined various approaches to achieve the

† Southern Research Institute.
‡ Scripps Clinic and Research Foundation.

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